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# Identification of *Leishmania* at the species level with matrix-assisted laser desorption ionization time-of-flight mass spectrometry

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## Abstract

Matrix-assisted laser desorption ionization time-of-flightMALDI-TOF mass spectrometry (MS) is now widely recognized as a powerful tool with which to identify bacteria and fungi at the species level, and sometimes in a rapid and accurate manner. We report herein an approach to identify, at the species level, *Leishmania* promastigotes from *in vitro* culture. We first constructed a reference database of spectra including the main *Leishmania* species known to cause human leishmaniasis. Then, the performance of the reference database in identifying *Leishmania* promastigotes was tested on a panel of 69 isolates obtained from patients. Our approach correctly identified 66 of the 69 isolates tested at the species level with log (score) values superior to 2. Two *Leishmania* isolates yielded non-interpretable MALDI-TOF MS patterns, owing to low log (score) values. Only one *Leishmania* isolate of *Leishmania peruviana* was misidentified as the closely related species *Leishmania braziliensis*, with a log (score) of 2.399. MALDI-TOF MS is a promising approach, providing rapid and accurate identification of *Leishmania* from *in vitro* culture at the species level.

**Keywords:** Identification, *Leishmania*, MALDI-TOF MS, mass spectra, parasite
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## Introduction

Human leishmaniasis, caused by 21 species of the obligate intracellular protozoan *Leishmania*, is one of the most neglected tropical diseases according to the WHO, despite the 2 million new cases recorded yearly [1]. This vector-borne disease encompasses several clinical syndromes, ranging from self-healing cutaneous infection to life-threatening visceral infections, partly depending on the *Leishmania* species involved [2]. Knowledge of the species involved is critical for predicting the evolution and prognosis, and for determining the appropriate treatment. However, conventional diagnostic techniques, i.e. microscopic examination of Giemsa-stained smears of infected tissues, *in vitro* cultivation of clinical samples on diphasic NNN or axenic media, and *Leishmania* serology assays, do not allow diagnosis at the species level [3]. Identification at the species level requires molecular biology techniques such as PCR or sequencing. These molecular biology techniques remain at an experimental stage, and are confined to research care centres. The reference standard method for identifying *Leishmania* species, as recommended by the WHO, remains isoenzyme analysis, which is labour-intensive, costly, and confined to reference centres [1]. Thus, no rapid and simple method for the species identification of *Leishmania* currently exists.

For approximately 10 years, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) has been proposed as an alternative to conventional and routine identification methods in clinical microbiological

laboratories. This identification technique was originally adapted for the identification of prokaryotic organisms [4]. New developments have extended its use to a few eukaryotic organisms, such as yeasts and moulds, making this technique a straightforward, fast and reliable identification method for bacteria, yeasts and moulds in a cost-effective way [5–7]. However, MALDI-TOF MS has not yet been adapted for the identification of *Leishmania* or any other protozoan parasites. In this article, we show how we first constructed a reference mass spectra library (MSL) by using cultures of 56 well-characterized *Leishmania* isolates, and then tested it by identifying a panel of 69 isolates with a MALDI-TOF MS-based approach.

## Materials and Methods

### *Leishmania* isolates and culture

A panel (panel 1) of 56 isolates was used to construct a mass spectra library, and is summarized in Table 1. The isolates were provided and accurately identified with the isoenzyme method by the Centre National de reference des Leishmanioses (CNR-L) (French National Reference Centre for Leishmaniasis) in Montpellier, France. A different panel (panel 2) of 69 isolates was used to assess identification performance (Table 2). These isolates were provided by the CNR-L, the Institut Pasteur of Alger, Algeria, and the Institut

**TABLE 1. Main characteristics of the *Leishmania* isolates furnished by the Centre National de reference des Leishmanioses included in the reference database**

| Taxon | Isolate | Provenance | Host | CF | Zymodem |
|---|---|---|---|---|---|
| *Leishmania infantum* | MHOM/EG/87/RTC2 | Egypt | *Homo sapiens* | VL | MON-98 |
| *L. infantum* | MHOM/FR/87/RM1 | France | *Canis familiaris* | VL | MON-108 |
| *L. infantum* | MHOM/TN/80/IPT1 | Tunisia | *H. sapiens* | VL | MON-1 |
| *L. infantum* | MHOM/DZ/82/LIPA59 | Algeria | *H. sapiens* | CL | MON-24 |
| *L. infantum* | MHOM/FR/78/LEM75 | France | *H. sapiens* | VL | MON-1 |
| *Leishmania donovani* | MHOM/IN/00/DEVI | India | *H. sapiens* | VL | MON-2 |
| *L. donovani* | MHOM/IN/96/THAK57 | India | *H. sapiens* | VL | MON-2 |
| *L. donovani* | MHOM/SD/98/LEM3580 | Sudan | *H. sapiens* | VL | MON-18 |
| *L. donovani* | MHOM/ET/67/HU3 | Ethiopia | *H. sapiens* | VL | MON-18 |
| *L. donovani* | MHOM/IN/80/DD8 | India | *H. sapiens* | VL | MON-2 |
| *Leishmania archibaldi* | MHOM/ET/72/GEBRE1 | Ethiopia | *H. sapiens* | VL | MON-82 |
| *L. archibaldi* | MCAN/SD/98/LEM3556 | Sudan | *C. familiaris* | VL | MON-82 |
| *Leishmania major* | MHOM/SU/73/5-ASKH | Turkmenistan | *H. sapiens* | CL | MON-4 |
| *L. major* | MHOM/IL/81/FRIEDLINa | Israel | *H. sapiens* | CL | MON-103 |
| *L. major* | MHOM/TN/2001/LEM4286 | Tunisia | *H. sapiens* | CL | MON-25 |
| *L. major* | MHOM/MA/2003/LEM4685 | Morocco | *H. sapiens* | CL | MON-25 |
| *L. major* | MHOM/YE/76/LEM62 | Yemen | *H. sapiens* | CL | MON-26 |
| *Leishmania aethiopica* | MHOM/ET/72/L100 | Ethiopia | *H. sapiens* | CL | MON-14 |
| *L. aethiopica* | MHOM/ET/91/KASSAYE | Ethiopia | *H. sapiens* | DCL | MON-248 |
| *Leishmania killicki* | MHOM/TN/80/LEM163 | Tunisia | *H. sapiens* | CL | MON-8 |
| *L. killicki* | MHOM/TN/80/LEM180 | Tunisia | *H. sapiens* | CL | MON-8 |
| *Leishmania tropica* | MHOM/MA/95/LEM3015 | Morocco | *H. sapiens* | CL | MON-264 |
| *L. tropica* | MHOM/SU/74/K27 | Azerbaijan | *H. sapiens* | CL | MON-60 |
| *L. tropica* | MHOM/GR/80/GR-L35 | Greece | *H. sapiens* | CL | MON-56 |
| *L. tropica* | 0000/00/84/LEM643 | NA | NA | VL | MON-104 |
| *L. tropica* | MRAT/IQ/72/ADHANIS1 | Iraq | *Rattus rattus* | VL | MON-5 |
| *Leishmania braziliensis* | MHOM/BR/75/M2904 | Brazil | *H. sapiens* | CL | MON-165 |
| *L. braziliensis* | MHOM/BR/84/LTB300 | Brazil | *H. sapiens* | MCL | MON-166 |
| *L. braziliensis* | MHOM/BR/82/LTB12JULY82 | Brazil | *H. sapiens* | MCL | MON-208 |
| *L. braziliensis* | MHOM/BR/75/M2903b | Brazil | *H. sapiens* | CL | MON-43 |
| *L. braziliensis* | MHOM/GF/2003/LAV008 | FG | *H. sapiens* | CL | MON-43 |
| *Leishmania guyanensis* | MHOM/GF/98/LEM3657 | FG | *H. sapiens* | CL | MON-45 |
| *L. guyanensis* | MHOM/GF/2003/LEM4570 | FG | *H. sapiens* | CL | MON-45 |
| *L. guyanensis* | MHOM/GF/2004/LAV016 | FG | *H. sapiens* | CL | MON-45 |
| *L. guyanensis* | MHOM/GF/79/LEM85 | FG | *H. sapiens* | CL | MON-45 |
| *Leishmania panamensis* | MHOM/EC/90/CALDERON | Equador | *H. sapiens* | CL | MON-161 |
| *L. panamensis* | MDAS/PA/71/LS94 | Panama | *H. sapiens* | CL | MON-47 |
| *Leishmania peruviana* | MHOM/PE/84/CE49 | Peru | *H. sapiens* | CL | MON-140 |
| *Leishmania lainsoni* | IUBI/BR/00/M12025 | Brazil | *Lutzomyia ubiquitalis* | None | MON-150 |
| *L. lainsoni* | MCUN/BR/85/M9342 | Brazil | *Cuniculus paca* | None | MON-151 |
| *Leishmania naiffi* | MDAS/BR/79/M5533 | Brazil | *Dasypus novemcinctus* | None | MON-148 |
| *Leishmania amazonensis* | MHOM/BR/72/M1845 | Brazil | *Proechimys* sp. | None | MON-41 |
| *L. amazonensis* | MHOM/BR/73/M2269 | Brazil | *H. sapiens* | CL | MON-132 |
| *L. amazonensis garnhami* | MHOM/VE/76/JAP78 | Venezuela | *H. sapiens* | CL | MON-41 |
| *Leishmania mexicana* | MNYC/BZ/62/M379 | Belize | *Nyctomys sumichrasti* | CL | MON-40 |
| *L. mexicana* | MHOM/BZ/82/BEL21 | Belize | *H. sapiens* | CL | MON-156 |
| *L. mexicana pifanoi* | MHOM/VE/57/LL1 | Venezuela | *H. sapiens* | DCL | MON-40 |
| *Leishmania pifanoi* | MHOM/VE/57/LL1a | Venezuela | *H. sapiens* | DCL | MON-40 |
| *Leishmania deanei* | MCOE/BR/78/M5088 | Brazil | *Coendou* sp. | None | MON-52 |
| *L. deanei* | MCOE/BR/74/M2674 | Brazil | *Coendou prehensilis* | CL | MON-134 |
| *Leishmania arabica* | MPSA/SA/83/JISH220 | Saudi Arabia | *Psammomys obesus* | VL | MON-99 |
| *Leishmania enriettii* | MCAV/BR/45/L88 | Brazil | *Cavia porcellus* | CL | MON-97 |
| *L. enriettii* | MCAV/BR/95/CUR2 | Brazil | *C. porcellus* | CL | MON-97 |
| *Leishmania gerbilli* | MRHO/CN/60/GERBILLI | China | *Rhombomys opimus* | CL | MON-22 |
| *Leishmania turanica* | MRHO/SU/65/VL | Turkmenistan | *R. opimus* | VL | MON-21 |
| *L. turanica* | MMEL/SU/79/MEL | Georgia | *Meles meles* | None | MON-65 |

CL, cutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis; FG, French Guyana; MCL, mucocutaneous leishmaniasis; NA, not available; VL, visceral leishmaniasis.

Pasteur of Tunis (PI-T), Tunisia (Table 2). The isolates were identified by isoenzymatic identification or by restriction fragment length polymorphism–PCR (RFLP-PCR) [8]. Only one isolate (L-68) was identified by RNA polymerase II gene sequencing, as described by Croan *et al.* [9]. Isoenzymatic identification was performed by the CNR-L in Montpellier,

**TABLE 2. Identification results obtained by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) in comparison with the isoenzymatic identification for the 69 blind-tested *Leishmania* samples (incorrect MALDI-TOF MS results are indicated in bold)**

| | | | | | Reference identification | | MALDI-TOF MS | |
|---|---|---|---|---|---|---|---|---|
| **Isolate** | **Source** | **Provenance** | **Host** | **Clinical form** | **Species ID** | **Method** | **Species ID** | **LS value** |
| L-1 | PI-T | Tunisia | *H.s* | VL | *Leishmania infantum* | RFLP-PCR | *L. infantum* | 2.702 |
| L-2 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.527 |
| L-3 | PI-A | Algeria | *H.s* | CL | *Leishmania killicki* | IsoE | *L. killicki* | 2.53 |
| L-4 | CNR-L | France | *H.s* | CL | *L. infantum* | IsoE | *L. infantum* | 2.56 |
| L-5 | PI-A | Algeria | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.616 |
| L-6 | PI-A | Algeria | *H.s* | CL | *Leishmania major* | IsoE | *L. major* | 2.578 |
| L-7 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.583 |
| L-8 | CNR-L | NA | *H.s* | CL | *L. infantum* | IsoE | *L. infantum* | 2.455 |
| L-9 | CNR-L | France | *H.s* | CL | *L. infantum* | IsoE | *L. infantum* | 2.532 |
| L-10 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.587 |
| L-11 | PI-A | Algeria | *H.s* | CL | *L. major* | IsoE | Non-interpretable | **1.575** |
| L-12 | PI-T | Tunisia | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.573 |
| L-13 | PI-A | Tunisia | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.616 |
| L-14 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.584 |
| L-15 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.513 |
| L-16 | CNR-L | Peru | *H.s* | CL | *Leishmania peruviana* | IsoE | ***Leishmania braziliensis*** | **2.399** |
| L-17 | CNR-L | Tunisia | *H.s* | CL | *L. infantum* | IsoE | *L. infantum* | 2.715 |
| L-18 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.351 |
| L-19 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.553 |
| L-20 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.499 |
| L-21 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.45 |
| L-22 | PI-T | Tunisia | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.721 |
| L-23 | PI-T | Tunisia | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.678 |
| L-24 | PI-A | Algeria | *H.s* | CL | *L. infantum* | IsoE | *L. infantum* | 2.676 |
| L-25 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.582 |
| L-26 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.614 |
| L-27 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.523 |
| L-28 | PI-A | Algeria | *H.s* | CL | *L. major* | IsoE | *L. major* | 2.68 |
| L-29 | PI-T | Tunisia | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.665 |
| L-30 | PI-A | Algeria | *H.s* | CL | *L. major* | IsoE | Non-interpretable | **1.737** |
| L-31 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.53 |
| L-32 | PI-A | Algeria | *H.s* | CL | *L. major* | IsoE | *L. major* | 2.675 |
| L-33 | PI-A | Algeria | *H.s* | CL | *L. major* | IsoE | *L. major* | 2.644 |
| L-34 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.548 |
| L-35 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.581 |
| L-36 | CNR-L | France | *H.s* | CL | *L. infantum* | IsoE | *L. infantum* | 2.459 |
| L-37 | PI-A | Algeria | *P.o* | None | *L. major* | IsoE | *L. major* | 2.609 |
| L-38 | PI-T | Tunisia | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.684 |
| L-39 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.533 |
| L-40 | PI-A | Algeria | *H.s* | CL | *L. killicki* | IsoE | *L. killicki* | 2.461 |
| L-41 | CNR-L | Algeria | *H.s* | CL | *L. infantum* | IsoE | *L. infantum* | 2.384 |
| L-42 | PI-A | Algeria | *H.s* | CL | *L. major* | IsoE | *L. major* | 2.644 |
| L-43 | CNR-L | Algeria | *H.s* | CL | *L. infantum* | IsoE | *L. infantum* | 2.609 |
| L-44 | PI-T | Tunisia | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.588 |
| L-45 | PI-T | Tunisia | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.639 |
| L-46 | PI-T | Tunisia | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.638 |
| L-47 | CNR-L | Algeria | *H.s* | CL | *L. infantum* | IsoE | *L. infantum* | 2.541 |
| L-48 | PI-A | Algeria | *H.s* | CL | *L. infantum* | RFLP-PCR | *L. infantum* | 2.627 |
| L-49 | PI-A | Algeria | *H.s* | CL | *L. infantum* | IsoE | *L. infantum* | 2.726 |
| L-50 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.53 |
| L-51 | PI-A | Tunisia | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.62 |
| L-52 | PI-A | Algeria | *H.s* | CL | *L. infantum* | RFLP-PCR | *L. infantum* | 2.62 |
| L-53 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.533 |
| L-54 | CNR-L | France | *H.s* | VL | *L. infantum* | IsoE | *L. infantum* | 2.579 |
| L-55 | CNR-L | French Guyana | *H.s* | CL | *L. braziliensis* | IsoE | *L. braziliensis* | 2.44 |
| L-56 | CNR-L | Colombia | *H.s* | CL | *Leishmania amazonensis* | IsoE | *L. amazonensis* | 2.594 |
| L-57 | CNR-L | Venezuela | *H.s* | DCL | *L. amazonensis* | IsoE | *L. amazonensis* | 2.468 |
| L-58 | CNR-L | French Guyana | *H.s* | CL | *Leishmania guyanensis* | IsoE | *L. guyanensis* | 2.52 |
| L-59 | CNR-L | French Guyana | *H.s* | CL | *L. guyanensis* | IsoE | *L. guyanensis* | 2.495 |
| L-60 | CNR-L | Belize | *H.s* | CL | *Leishmania mexicana* | IsoE | *L. mexicana* | 2.503 |
| L-61 | CNR-L | Ethiopia | *H.s* | CL | *Leishmania aethiopica* | IsoE | *L. aethiopica* | 2.209 |
| L-62 | CNR-L | Iran | *H.s* | CL | *Leishmania tropica* | IsoE | *L. tropica* | 2.465 |
| L-63 | CNR-L | India | *H.s* | VL | *Leishmania donovani* | IsoE | *L. donovani* | 2.438 |
| L-64 | CNR-L | Brazil | *C.f* | MCL | *L. braziliensis* | IsoE | *L. braziliensis* | 2.249 |
| L-65 | CNR-L | Bolivia | *H.s* | ML | *L. braziliensis* | IsoE | *L. braziliensis* | 2.456 |
| L-66 | CNR-L | Mexico | *H.s* | DCL | *L. mexicana* | IsoE | *L. mexicana* | 2.381 |
| L-67 | CNR-L | Costa Rica | *H.s* | DCL | *L. guyanensis* | IsoE | *L. guyanensis* | 2.186 |
| L-68 | CNR-L | Peru | *H.s* | CL | *Leishmania lainsoni* | RNAIIpol | *L. lainsoni* | 2.061 |
| L-69 | CNR-L | Syria | *H.s* | CL | *L. tropica* | IsoE | *L. tropica* | 2.417 |

*C.f*, *Canis familiaris*; CL, cutaneous leishmaniasis; CNR-L, Centre National de Référence des Leishmanioses, Montpellier, France; DCL, diffuse cutaneous leishmaniasis; *H.s*, *Homo sapiens*; IsoE, isoenzymatic identification; LS, log (score); MCL, mucocutaneous leishmaniasis; ML, mucosa leishmaniasis; NA, not available; PI-A, Institut Pasteur of Alger; PI-T, Institut Pasteur of Tunis, *P.o*, *Psammomys obesus*; RFLP-PCR, restriction fragment length polymorphism–PCR; RNAIIpol, RNA II polymerase gene sequencing; VL, visceral leishmaniasis.

France, and the Institut Pasteur of Alger, Algeria as described by Rioux *et al.* [10]. Identification by RFLP-PCR was performed by the PI-T as described by Schönian *et al.* [8]. The 'reference identification' of isolates represented isoenzymatic identification, when available; otherwise the 'reference identification' represented the result of the identification via RFLP-PCR.

All isolates were cryoconserved in liquid nitrogen in a tube containing RPMI medium supplemented with 30% fetal bovine serum and 20% dimethylsulphoxide at a concentration of approximately 3 million promastigotes per millilitre. Cultures were performed as follows. The cryoconserved tubes were rapidly thawed in a 37°C water bath, and immediately washed three times with 10 mL of RPMI medium (RPMI Medium 1640 + 2 mM L-glutamine + 25 mM HEPES; Gibco BRL, Life Technologies SAS, Saint Aubin, France). Then, the pellet of promastigotes was suspended in 2 mL of RPMI medium supplemented with 15% decomplemented fetal bovine serum, 100 UI/mg/L penicillin/streptomycin solution (Gibco BRL), and 100 mg/L kanamycin (Gibco BRL). Eight drops (*c.* 20 μL/drop) of this suspension were inoculated in a traditional culture tube containing Novy–MacNeal–Nicolle medium and incubated for 1 week at 25°C. Then, five drops of the culture supernatant were transferred to RPMI medium as described by Gouzelou *et al.* [11], and incubated for 1 week at 25°C. After 7 days of growth, promastigotes were harvested by centrifugation (10 min at 3000°**g**) of the RPMI medium, and then washed three times with 10 mL of 0.9% NaCl. Then, the promastigote pellet was gently suspended in one drop (approximately 20 μL) of 0.9% NaCl.

#### Mass spectra acquisition

A 1-μL drop of the saline solution of promastigotes was deposited on a spot of a polished steel target (MTP384 polished steel target; Bruker Daltonics, Bremen, Germany) and air-dried. The *Leishmania* isolates used to construct the mass spectra library were deposited in decaplicate, whereas the *Leishmania* isolates used to assess identification performance were deposited in quadruplicate. Each spot was then covered with 1 μL of the matrix solution. The matrix solution was a daily prepared saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid (Sigma-Aldrich, Lyon, France). The spectra were acquired after 240 shots in linear mode with a Microflex LT instrument (Bruker Daltonics) in the ion-positive mode with a 337-nm nitrogen laser. The following adjustments were used: delay, 170 ns; ion source 1 voltage, 20 kV; ion source 2 voltage, 18.5 kV; and mass range, 3–20 kDa. The data were automatically acquired by the AutoXecute of the FlexControl v2.4 software, and exported into MaldiBiotyper v2.1 software. Only peaks with a signal/noise ratio of ≥3 were considered. A calibration was performed before and after each run with the Bruker Bacterial Standard Test calibrator (Bruker Daltonics). A negative extraction control was spotted on each run to detect contamination.

#### Evaluation of the mass spectra quality

With FlexAnalysis 3.3.80.0 software (Bruker Daltonics), the first spectra acquired for each of the 56 isolates (panel 1) was visualized and then analysed: the peak list (from 3 to 20 kDa) and the signal/noise ratio (>3) of peaks were recorded.

#### MSL construction

The MSL was composed of 56 reference mass spectra (MSPs). An MSP was created for each *Leishmania* isolate of panel 1 (Table 1). Each MSP was created by combining the results of ten raw spectra per *Leishmania* isolate with the 'MSP creation' function of the MaldiBiotyper software.

#### MSL validation

To assess the technical reproducibility of the spectra obtained with the same biological sample, the log (score) (LS) values of each raw spectrum composing an MSP were obtained by comparing each raw spectrum with its proper MSP by use of the 'Start identification' function of the MaldiBiotyper software. Moreover, to assess the biological reproducibility of the spectra obtained with each isolate, another subculture of each isolate was performed. The four raw spectra obtained from this subculture were blasted against the MSL with the 'Start identification' function of the MaldiBiotyper software. The identification result showing the best log (score) value among the four spots was recorded.

#### MALDI-TOF MS-based identification of *Leishmania* isolates

*Leishmania* isolates were identified in blind tests. Anonymous *Leishmania* culture samples were provided to the experimenter who performed the identification as follows. Each of the four raw spectra obtained for each *Leishmania* sample were identified by comparison with the MSL by use of the 'Start identification' function of the MaldiBiotyper software. The best match and its corresponding LS value were recorded for each of the four raw spectra. The MALDI-TOF MS-based identification of a *Leishmania* sample was considered to be interpretable if the raw spectra with the highest LS value matched with an MSP of our MSL with an LS value superior to 2. In the opposite instance, the MALDI-TOF MS-based identification was considered to be non-interpretable. An interpretable MALDI-TOF MS-based identification was considered to be correct if it matched with the isoenzymatic identification or the RFLP-PCR identification.

## Results

### Quality of the mass spectra

The 56 spectra showed different profiles and several peaks with good resolution, ranging from 3003.528 to 16154.79 Da (Fig. 1). Furthermore, the 56 spectra analysed were composed of an average of 67 ± 34 peaks per spectrum (ranging from 15 peaks to 161), with a signal/noise ratio ranging from 3 to 94.9 (mean = 8.57).

### MSP library validation

The LS values obtained by comparing each of the ten raw spectra composing an MSP with its proper MSP (technical reproducibility) ranged from 1.843 to 2.918, with a mean value of 2.753 ± 0.09. Only one raw spectrum of the 560 raw spectra gave an LS value of <2, the LS threshold value required to confirm good identification according to the manufacturer (Table S1).

Reproducibility was excellent, as the raw spectra with the highest LS values acquired from a different subculture of a isolate best matched with their proper MSP for 54 of the 56 reference isolates, with LS values ranging from 2.345 to 2.858 (mean = 2.700 ± 0.100). The two remaining subcultures of reference isolates best matched with an MSP of a different reference isolate but belonging to the same species, and even to the same zymodeme: the subculture of *Leishmania major* MHOM/MA/2003/LEM4685 (zymodeme MON-25) best matched with the MSP of the reference isolate *L. major* MHOM/TN/2001/LEM4286 (zymodeme MON-25), with an LS value of 2.671; and the subculture of *Leishmania mexicana* MNYC/BZ/62/M379 (zymodeme MON-40) best matched with the MSP of the reference isolate *L. mexicana* MHOM/VE/57/LL1 (zymodeme MON-40), with an LS value of 2.368.

### MALDI-TOF MS-based identification of *Leishmania* samples

Of the 69 clinical isolates identified by MALDI-TOF MS in a blind test, 67 isolates gave interpretable MALDI-TOF MS-based identification results; that is, the best match of four spots had an LS value of >2. The LS values of these 67 isolates ranged from 2.061 to 2.726 (mean = 2.536 ± 0.13). Sixty-six of these 67 (95.65% of all isolates) interpretable MALDI-TOF MS results were correct; that is, the MALDI-TOF MS identification was concordant with the isoenzymatic identification or the RFLP-PCR identification at the species level.

Only *Leishmania peruviana* L-16 was misidentified, as *Leishmania braziliensis*, with an LS value of 2.399. This sample matched with the reference of *L. peruviana* with an LS value of only 2.075.

The MALDI-TOF MS identification was non-interpretable for the *L. major* isolates L-11 and L-30, because the best match of the four spots did not reach the threshold of 2 (LS values of 1.575 and 1.737, respectively).

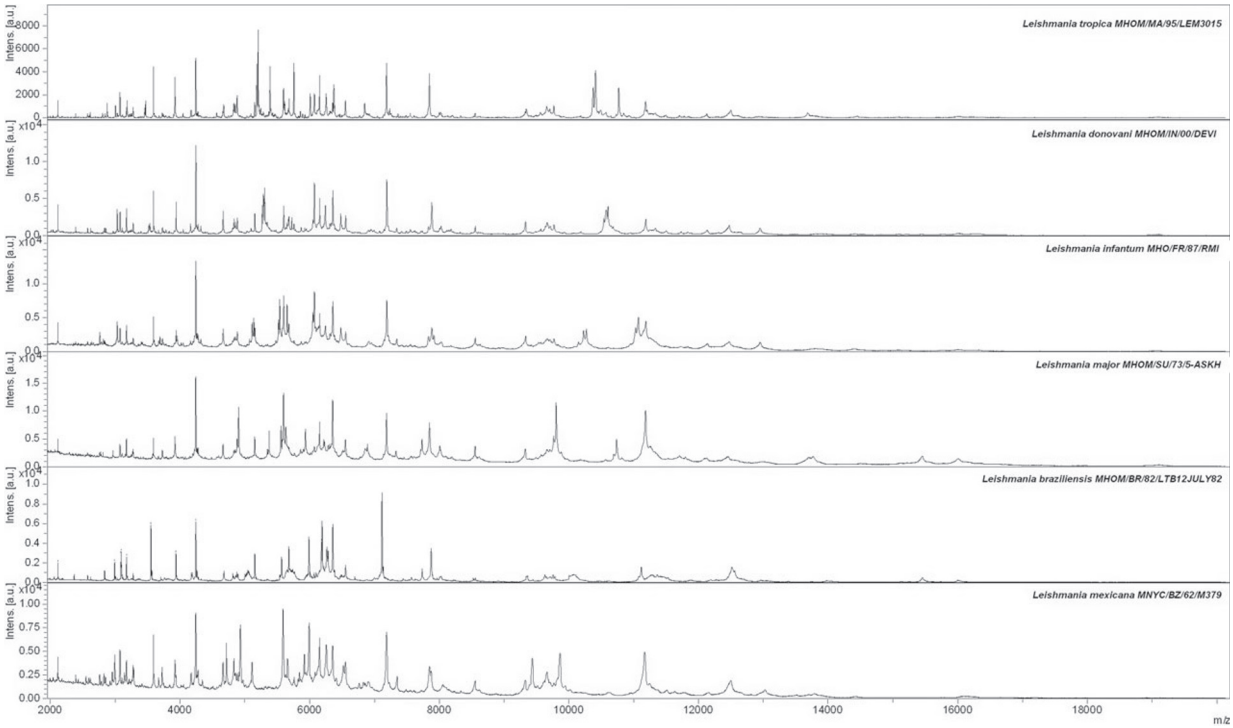


**FIG. 1.** Mass spectra of six different isolates of *Leishmania* included in the reference library.

## Discussion

MALDI-TOF MS is now well adapted to the identification of prokaryotes such as bacteria [4], as well as of eukaryotic unicellular organisms such as yeasts [6]. More recently, the use of MALDI-TOF MS-based assays was extended to identify other organisms, such as moulds, dermatophytes, and even insects [7,12–13]. However, MALDI-TOF MS has not yet been used to identify protozoan parasites. This study shows that this is possible for cultivated *Leishmania* promastigotes.

We deliberately used a simple protocol consisting of only washing a rich culture of promastigotes with NaCl. Williams *et al.* [14] reported that spectra acquired from whole bacterial cells treated with 0.9% NaCl were of poor quality as compared with those acquired from whole bacterial cells treated with de-ionized water or ethanol. However, de-ionized water lyses promastigote cells, leading to the lost of cellular contents. Additionally, our experiments showed that the spectra obtained from the saline solution of promastigotes were composed of numerous peaks (ranging from 15 to 161) with high resolution and intensity, encompassing a large mass/charge interval (from 3 kDa to approximately 16 kDa). According to Erhard *et al.*, the quality of the promastigote spectra obtained was sufficient to ensure identification at the species level, as the spectra clearly exceeded the 17-peak threshold, thereby ensuring the species specificity of a spectrum. The good quality of promastigote spectra in saline solution could be explained by the relatively low signal emitted by the 0.9% NaCl as compared with the signals emitted by promastigote cells.

Moreover, this simple protocol provided highly reproducible spectra. Indeed, technical reproducibility experiments led to LS values as high as $2.753 \pm 0.09$. Data concerning the technical reproducibility are scarce in the microbiology literature. The only available data that we found for eukaryotes were for filamentous fungi. Cassagne *et al.* [7] reported technical reproducibility with an LS value of approximately 2.5 when comparing the raw spectra composing an MSP of a vast panel of moulds with its proper MSP. The reproducibility results obtained here are superior probably because *Leishmania* promastigotes do not show the phenetic polymorphism that is commonly found with cultured moulds.

On this basis, we constructed a 56-reference isolate database, which proved to be able to identify, at the zymodeme level, a subculture of all of the isolates represented in the reference database. Indeed, 54 of the 56 subcultures of the reference isolates matched with their proper MSP (mean LS value of $2.700 \pm 0.10$), whereas the two remaining subcultures best matched with an MSP corresponding to another reference belonging to the same zymodeme.

When testing our MALDI-TOF MS method for the identification of isolates, we found that 95.65% of the blindly tested samples were correctly identified. The results were not interpretable for only two samples, even after a second identification test. Otherwise, only one isolate was misidentified. This identification rate is similar to, and in some cases even better than, those obtained for other microorganisms. Seng *et al.* reported an identification rate at the species level of 84.1% for clinical bacterial isolates, Cassagne *et al.* reported a rate of >85% for clinical isolates, and De Carolis *et al.* [15] reported a rate of 96.8% for a few species of mould [7]. Even the isolates belonging to species represented in the reference library by only one or two references, such as *Leishmania lainsoni*, *Leishmania aethiopica*, and *Leishmania killicki*, were correctly identified (with the exception of the misidentified isolate of *L. peruviana*). Notably, the LS values that we obtained when identifying the clinical *Leishmania* samples were particularly high, with a mean of 2.536, which is far above the values obtained with other eukaryotic organisms, such as filamentous fungi (mean = 2.206) [15] and yeasts (mean = 2.225) [16].

Only one *L. peruviana* isolate was misidentified as *L. braziliensis*, with a high LS value (2.399). *L. peruviana* and *L. braziliensis* are two closely related species belonging to the *Viannia* subgenus, and *L. peruviana* is included in the *L. braziliensis* complex [17]. For example, isolates of these two species show very similar multilocus enzyme electrophoresis profiles, which can only be distinguished by the use of mannose phosphate isomerase [18]. Furthermore, it has been shown that the sequence of the gene encoding mannose phosphate isomerase differs between these two species by a single nucleotide, leading to a change from threonine (*L. braziliensis*) to arginine (*L. peruviana*) [18]. The lack of MALDI-TOF MS-based discrimination between these species is therefore not surprising.

Overall, this study has shown that MALDI-TOF MS is a promising approach with which to identify *Leishmania* from *in vitro* cultures with high identification rates and LS values. The database that we have built up here constitutes a first prototype that needs to be enlarged in order to increase its performance. A limitation of the technique for clinical diagnostic use is the need for cultivated parasites. Nevertheless, as compared with molecular biology, this approach offers great advantages, in particular speed, simplicity, and cost, for isolate identification at the species level.

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## Transparency Declaration

The authors declare no conflicts of interest.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Log (score) values of each raw spectrum composing the 56-reference mass spectrum included in the mass spectra library

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